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Influenza A virus transactivates the mouse envelope gene encoding syncytin B and its regulator, glial cells missing 1

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Recently, two candidate analogs for human syncytin, denoted syncytins A and B, were identified in the murine genome. These were found to have expression patterns and functions similar to human syncytin. In addition, the identification of glial cells missing (GCM)-binding motifs in putative promoter regions of the mouse syncytins imply analogous regulation. Transcriptional modulation of syncytin by exogenous agents was recently suggested by studies reporting transactivation of syncytin in human cell lines following virus infections. The authors report that influenza A virus infection increased the levels of transcripts encoding Gcm1 and syncytin B, but not syncytin A, in NIH-3T3 cells as well as in mouse primary neurons or glia. Overexpression of human GCM1 in NIH-3T3 cells resulted in increased levels of transcripts encoding syncytin B but not syncytin A. Systemic administration of neurotropic influenza A virus resulted in a neuronal infection and increased levels of Gcm1-encoding transcripts in brains of young mice. The mouse may therefore be useful for studies on the expression and function of endogenous retroviral envelope genes and transcription factors regulating their expression in the placenta and brain during physiological or pathological conditions. *Journal of NeuroVirology* (2007) **13,** 29–37.

Keywords: brain; gene expression; placenta; virus infection

Introduction

A human endogenous retrovirus (HERV) element in the W family on chromosome 7q21.1 contains an intact *env* gene highly expressed in the human placenta, where it has been proposed to play an important role in the formation of the syncytiotrophoblast cell layer (Blond *et al*, 2000; Voisset *et al*, 1999, 2000). In light of its fusogenic properties, mediated via binding to the ASCT1 and ASCT2 amino acid transporters (Blond *et al*, 2000; Lavillette *et al*, 2002), the protein has been denoted syncytin (Mi *et al*, 2000). Expression of syncytin appears to be more or less restricted to the human placenta, although it has also been observed in microglia of normal human brains (Perron *et al*, 2005).

Reduced levels of syncytin transcripts and protein have been observed in placentas from pregnancies complicated by preeclampsia (Keith *et al*, 2002; Knerr *et al*, 2002; Kudo *et al*, 2003; Lee *et al*, 2001). Increased expression of syncytin has been observed in microglia/macrophages in acutely demyelinating plaques in brains from patients with multiple sclerosis (Antony *et al*, 2004; Perron *et al*, 2005). Antony and coworkers (Antony *et al*, 2004) further reported that ectopic expression of syncytin in the murine corpus callosum induced oligodendrocyte death and caused neurobehavioral deficits reminiscent of those observed in patients with multiple sclerosis. Ruprecht and coworkers (Ruprecht *et al*, 2006) recently reported induction of a HERV-W envelope protein, likely representing syncytin, by herpes

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simplex type 1 virus. In addition, we reported increased levels of transcripts encoding syncytin following influenza A virus infection in different human cell-lines (Nellaker *et al*, 2006). Furthermore, signs of mitochondrial toxicity were observed in cells overexpressing syncytin. Taken together, these reports suggest that a normal, high-level expression of syncytin in syncytiotrophoblasts is crucial for placental development and functioning, whereas its induction in extraplacental tissues may adversely affect cellular functions.

Studies on the transcriptional regulation of the gene encoding syncytin have identified cyclic adenosine monophosphate (cAMP)-responsive elements in a promoter region (U3) of the 5′ long terminal repeat (LTR) and an upstream, trophoblast-specific enhancer element (Prudhomme *et al*, 2004). The transcription factor glial cells missing (GCM)1 has been reported to enhance expression of syncytin-encoding transcripts in cell types derived from the human placenta and has been suggested to mediate a tissue-specific, high level expression in this organ (Cheng and Handwerger, 2005; Prudhomme *et al*, 2004; Yu *et al*, 2002).

It is not known if other mammals in which trophoblast fusion occurs (e.g., mice) have also adopted retroviral envelopes. Recently, Dupressoir and coworkers (Dupressoir *et al*, 2005) identified two open reading frames encoding retroviral envelope proteins, denoted syncytins A and B, in the mouse genome. These were found to be expressed mainly in the mouse placenta and were found to efficiently fuse certain cell types when overexpressed *in vitro*. Although phylogenetically unrelated to human syncytin, these murine envelope proteins are thus good candidate analogs of human syncytin. This notion is further supported by the alleged identification of GCM-binding motifs in putative promoter regions of both of these genes (Dupressoir *et al*, 2005), implying analogous regulation. The importance of Gcm1 for placental development in the mouse is supported by the finding that mice lacking Gcm1 die between gestational day 9.5 and 10 due to failure to develop a functional syncytiotrophoblast layer (Cross *et al*, 2002; Schreiber *et al*, 2000).

Human GCM1 and mouse Gcm1 are orthologs of *gcm/glide* identified in *Drosophila*, in which *gcm* is expressed in all embryonic glia except mesoderm-derived midline glia. *Gcm* mutants lack lateral glia, whereas ectopic expression of *gcm* in neurons causes glial transformation (Hosoya *et al*, 1995; Jones *et al*, 1995). A corresponding role in the mammalian brain was suggested by Iwasaki *et al* (2003), who reported that overexpression of mouse Gcm1 in mouse fibroblasts induced expression of the astrocyte marker S100b. Furthermore, they reported the induction of gliogenesis by Gcm1 both *in vitro* and *in vivo*.

Taken together, these studies suggest the existence of endogenous retroviral *env* genes in the mouse genome, with high levels of expression in the mouse placenta, potentially mediated by Gcm1. If so, the mouse may be used as a model to study the regulation and potential roles of such genes in the placenta and brain during physiological or pathological conditions.

In the present study we investigated the influence of influenza A virus infections on the transcription of the genes encoding mouse Gcm1, syncytin A, and syncytin B *in vitro* and *in vivo*. Furthermore, we investigated if Gcm1 is involved in the transcriptional regulation of syncytin A and/or syncytin B.

Results

Influenza A virus infections in vitro

Twenty-four hours after infection with 0.5 multiples of infection (MOI) of influenza A/WSN/33 virus, NIH-3T3 cells contained 9.1-fold elevated levels of transcripts encoding Gcm1 and 3.7-fold elevated levels of transcripts encoding syncytin B as compared to baseline conditions. The relative levels of transcripts encoding syncytin A were however not affected by the infection (Figure 1A). Because these observations were made in a mouse fibroblast cell line, we next determined the influence of the influenza A virus infection on the expression of the genes encoding Gcm1, syncytin A, and syncytin B in primary cells derived from mouse brain.

Primary cultures of mouse hippocampus neurons or cortical glial cells were inoculated with 0.5 MOI of influenza A/WSN/33 virus. Similarly to what we observed in the NIH-3T3 cells, both of these cell types responded with increased levels of transcripts encoding Gcm1 and syncytin B, whereas the levels of transcripts encoding syncytin A remained at levels similar to those observed in uninfected control cultures (Figure 1B, C). Thus, the levels of transcripts encoding Gcm1 appear to vary concurrently to those encoding syncytin B.

Transfection of NIH-3T3 cells with human GCM1

To investigate if Gcm1 mediates syncytin B expression, NIH-3T3 cells were transfected with a plasmid construct containing the open reading frame encoding human GCM1 controlled by the immediate early cytomegalovirus promoter. Because the promoter for human syncytin has been reported to be responsive to cAMP-dependent factors (Knerr *et al*, 2005; Mi *et al*, 2000; Prudhomme *et al*, 2004), GCM1-transfected cells and control cells transfected with an expression plasmid encoding enhanced green fluorescent protein were cultured in the presence or absence of forskolin, an activator of adenylate cyclase, resulting in elevated cellular levels of cAMP (Seamon and Daly, 1981). In light of the previous identification of *S100b* being a target gene for Gcm1 (Iwasaki *et al*, 2003), we determined the levels of such gene transcripts in these cultures. As expected, cells overexpressing GCM1 contained considerably higher levels

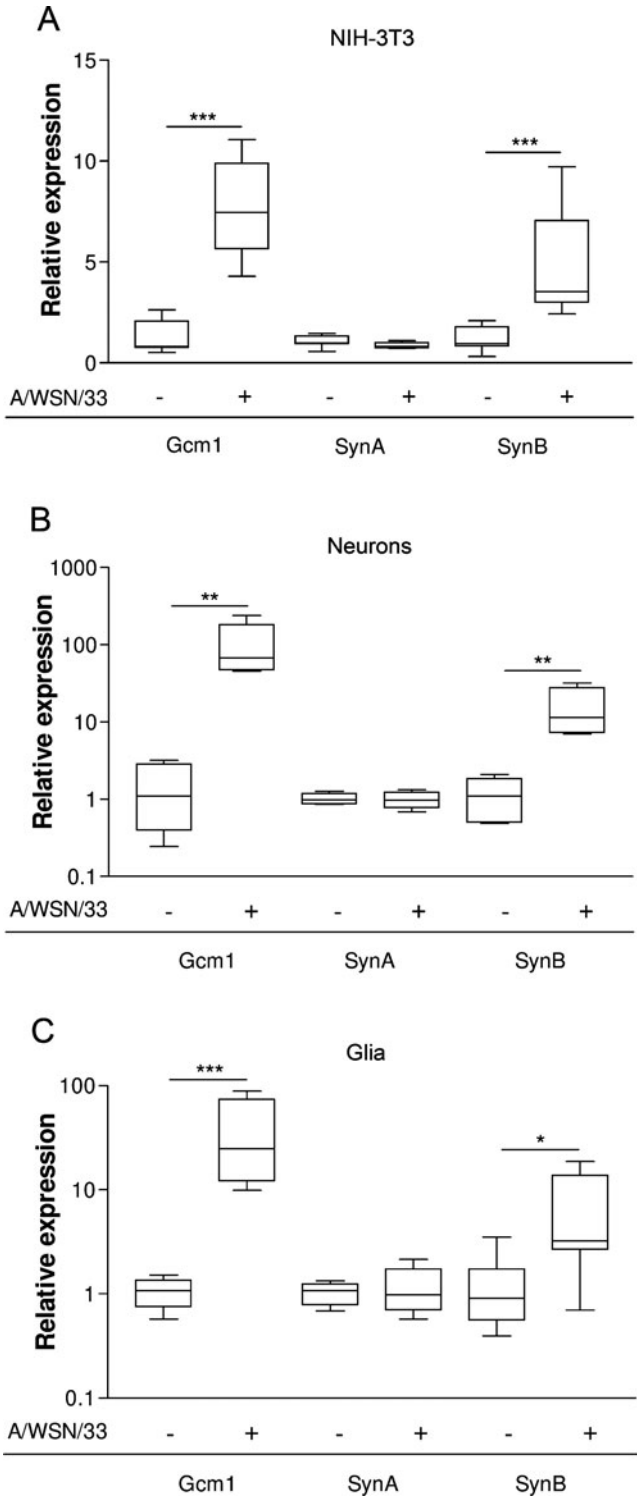

Figure 1 Levels of transcripts in influenza A/WSN/33 infected NIH-3T3 cells and primary cells. The levels of transcripts encoding Gcm1, syncytin A, and syncytin B in virus infected NIH-3T3 cells (**A**), primary neurons (**B**), and primary glial cells (**C**) are shown. The levels of transcripts in infected cells (+) are normalized to those observed in control cells (−). The box-and-whisker plots represent data from six to eight cultures in each group. The box extends from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and lowest values. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

of *S100b* transcripts than did control cells (21-fold). Although forskolin treatment alone of the NIH-3T3 cells did not affect the levels of *S100b* transcripts, the combination of forskolin treatment and GCM1 overexpression further, significantly, increased the levels of *S100b* transcripts to 37-fold the levels observed in control cultures (Figure 2A). In addition to the induction of *S100b* transcripts, overexpression of GCM1, but not forskolin treatment, resulted in a significant, 2.5-fold increase in the levels of transcripts encoding syncytin B (Figure 2B). These levels were further increased by forskolin, to 3.5-fold the levels observed in control cells, although the effect of forskolin was not significant when compared to transfected cells. The levels of transcripts encoding syncytin A were not affected by forskolin-treatment or by GCM1 overexpression (Figure 2C). The levels of syncytin A–encoding transcripts were, however, significantly elevated in transfected cells treated with forskolin as compared to forskolin-treated cells or GCM1-transfected cells (Figure 2C). Taken together, these results suggest that S100b and syncytin B are target genes for Gcm1. The effect of forskolin treatment of transfected cells on the activities of these genes and that encoding syncytin A support additional influences of cAMP-mediated events on their mRNA levels. Forskolin-treatment alone of the NIH-3T3 cells investigated did not detectably affect the endogenous levels of transcripts encoding Gcm1 as compared to control cells (data not shown).

Identification of GCM-binding motifs

Fifty-kilobase regions of the murine genome containing the *S100b* gene and the two open reading frames (ORFs) encoding the murine syncytins, respectively, were screened for the presence of GCM-binding motifs (Akiyama *et al*, 1996; Schreiber *et al*, 1997). Identified sites in these sequences are indicated by vertical lines in Figure 3. The positions of the *S100b* gene locus and the two open reading frames encoding syncytin A and syncytin B, respectively, are also indicated. In the 5′-flanking region of the *S100b* locus, we identified five GCM-binding sites located 6.9, 9.8, 13, 14, and 21 kb upstream of the transcriptional start. Three such sites were identified 5′ of the ORF encoding syncytin B: 1.3, 2.4, and 3.3 kb upstream of the translational start, whereas one such binding site was identified at 2.9 kb 5′ of the ORF encoding syncytin A.

mRNA levels in vivo

In their study, Dupressoir and coworkers (Dupressoir *et al*, 2005) investigated the levels of transcripts encoding syncytin A and syncytin B in different embryonic and adult mouse tissues, reporting high levels of both transcripts in placenta after gestational day 11.5. Appreciable expression of the two transcripts was not detected in other tissues with the exception of adult brain tissue, which contained detectable levels of transcripts encoding syncytin A

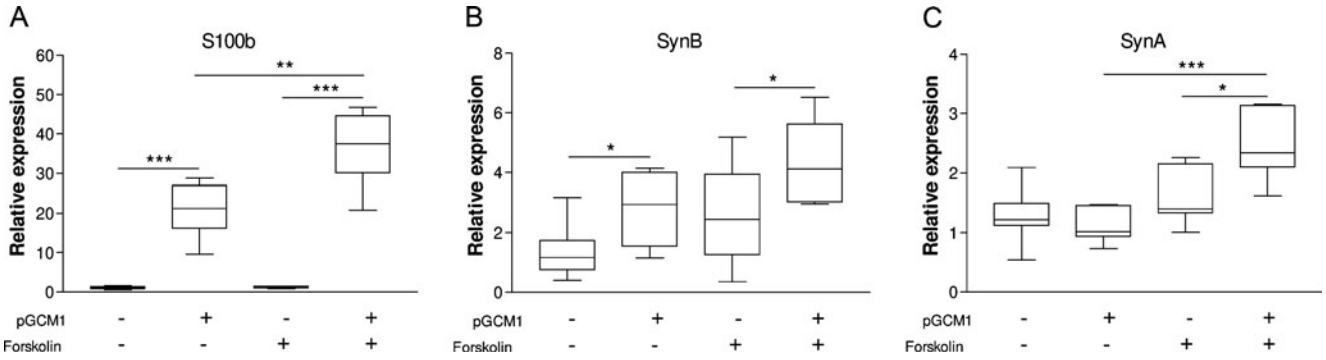

Figure 2 Levels of transcripts in transfected NIH-3T3 cells. The levels of transcripts encoding S100b (**A**), syncytin B (**B**), or syncytin A (**C**) in NIH-3T3 cells transfected with EGFP expression plasmid (controls), cells transfected with human GCM1, cells treated with forskolin, and cells transfected with human GCM1 in the presence of forskolin are shown. The levels of transcripts in each group are normalized to those observed in control cells. The box-and-whisker plots represent data from seven cultures in each group. The box extends from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and lowest values. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

(Dupressoir *et al*, 2005). Iwasaki and coworkers previously reported high levels of transcripts encoding Gcm1 in the mouse placenta, as well as detectable levels of such transcripts in embryonic mouse brains at midgestation after which the levels in the brain declined (Iwasaki *et al*, 2003). By quantitative real-time polymerase chain reaction (PCR), we determined the levels of transcripts encoding Gcm1, syncytin A, and syncytin B relative to those encoding glyceraldehyd-3-phosphate dehydrogenase (GAPDH) in whole brains from C57BL/6 mice of different ages, ranging from embryonal day (E)17 to day 24 of postnatal life (P24). For a comparison, the levels observed in E17 placentas were also determined. As can be seen from Figure 4, the highest levels of all three transcripts were detected in the placentas, with those encoding syncytin B present at approximately 8-fold higher levels than those encoding syncytin A. E17–P7 brains harbored readily detectable levels of transcripts encoding Gcm1, which declined substantially at later stages. Transcripts encoding syncytin A were detected at relatively high levels in the brain tissues, as compared to transcripts encoding Gcm1. These levels appeared fairly constant throughout the different ages investigated. Similar observations were made for transcripts encoding syncytin B, although these transcripts were present at approximately 40-fold lower levels than those encoding syncytin A. Analyses of the levels of the three different transcripts in dissected brain regions gave similar results: transcripts encoding syncytin A were detected at the highest levels in all three regions, followed by intermediate levels of syncytin B and low levels of transcripts encoding Gcm1, regardless of age (Figure 5A–C).

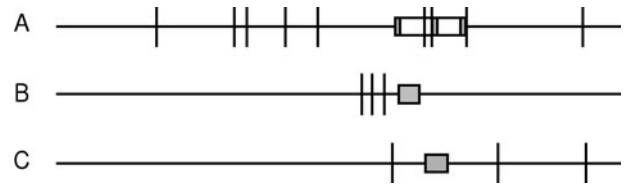

Figure 3 Identification of GCM-binding motifs. Fifty-kilobase regions of the murine genome containing the *S100b* gene (**A**), the ORF encoding syncytin B (**B**), and the ORF encoding syncytin A (**C**) are shown. GCM binding motifs are indicated by vertical lines. The exons and introns of the *S100b* gene are indicated by grey and white boxes, respectively. The ORFs encoding syncytins A and B are indicated by grey boxes.

Influenza A/WSN/33 virus infection of mice

In vivo, neurovirulent strains of influenza A virus can target the mouse brain with an ensuing neuronal infection following systemic administration (Reinacher *et al*, 1983). We thus investigated if the A/WSN/33 virus infection can target the brain and modulate the expression of Gcm1 *in vivo*. Three-day-old C57BL/6 mice were therefore injected intraperitoneally with 2400 plaque-forming units (PFU) of

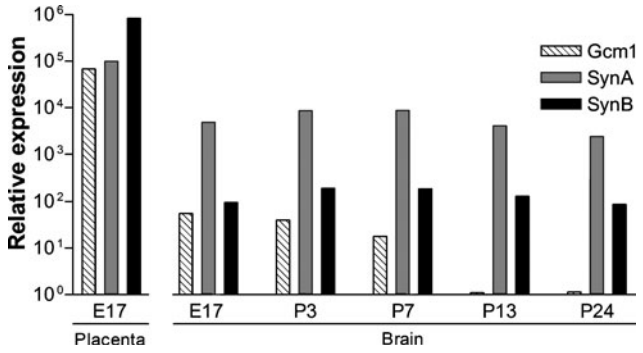

Figure 4 Levels of transcripts in brain and placenta. The average levels of transcripts encoding Gcm1, syncytin A, and syncytin B in brains from animals sampled at the following ages: embryonal day (E)17, postnatal day (P)3, P7, P13, and P24 ($n = 6$–9 in each group). For a comparison, the levels of these transcripts in the placenta at E17 ($n = 7$) are also indicated. In order to allow a comparison between the different transcripts, the levels of all transcripts are normalized to those encoding Gcm1 in brains at P13.

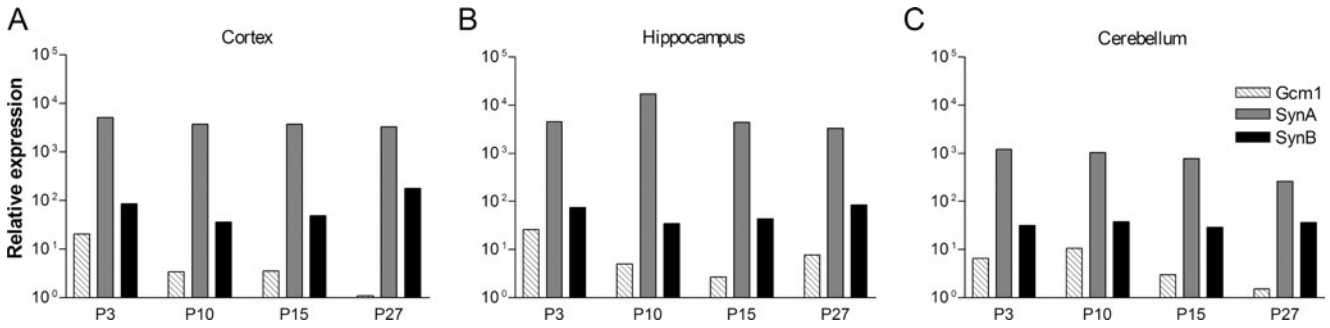

Figure 5 Levels of transcripts in brain regions. The level of transcripts encoding Gcm1, syncytin A, and syncytin B in cortex (**A**), hippocampus (**B**), and cerebellum (**C**) at the following ages: postnatal day (P)3, P10, P15, and P27 ($n = 2$). To allow a comparison across genes, ages and regions the levels of transcripts in each group are normalized to those observed for Gcm1 in cortex at P27.

influenza A/WSN/33 virus. Four days after the infection all mice survived. At this time point, viral RNA and antigens were detected in the brains of each of the infected animals, as determined by reverse transcriptase (RT)-PCR and immunohistochemistry (Figure 6A). The relative levels of transcripts encoding Gcm1 in these brains were elevated by 6.4-fold as compared to the levels observed in control mice injected with phosphate-buffered saline (PBS) (Figure 6B). The levels of transcripts encoding S100b, syncytin A, and syncytin B were, however, detected at levels similar to those observed in control animals (data not shown).

Discussion

In the present study, we report that an influenza A/WSN/33 virus infection triggers transcriptional activation of the gene encoding the transcription factor Gcm1 in NIH-3T3 cells as well as in primary cultures of mouse hippocampal neurons or cortical glia. In such infected cultures, transcripts encoding syncytin B were detected at elevated levels as compared to uninfected control cells. Similar findings regarding human syncytin were recently reported in different human cell lines following influenza A virus infection (Nellaker *et al*, 2006). Overexpression of human GCM1 in NIH-3T3 cells induced transcription of the gene encoding S100b, consistent with the previous findings by Iwasaki and coworkers (Iwasaki *et al*, 2003) and with the presence of five GCM-binding motifs within 21 kb upstream of the transcriptional start. Induction of transcripts encoding syncytin B in such transfected cells, albeit to a lesser degree, appears consistent with the identification of three GCM-binding motifs within 3.3 kb upstream of the open reading frame. The levels of transcripts encoding syncytin A were not affected by virus infection or by GCM1 overexpression in the NIH-3T3 cells, although we identified a single GCM-binding motif 2.9 kb upstream of the ORF. GCM binding sites may not be functional in all cell types as is the case in human nonplacental cells. In such cells, overexpression of GCM1 fails to induce syncytin transcription, although it is a functional enhancer of syncytin expression in choriocarcinoma cells (Yu *et al*, 2002). The lack of expression of syncytin in human nonplacental tissues has been suggested to be caused by heavy methylation of the upstream regulatory sequence in such tissues (Matouskova *et al*, 2006). The restricted expression of human syncytin (Blond *et al*, 2000; Mi *et al*, 2000), however, contrasts to that of mouse syncytin A, which was expressed in all cell types and tissues investigated here. Taken together, our observations in both virus-infected and GCM1-transfected cells therefore suggest that Gcm1 is involved in the transcriptional regulation of syncytin B. Our observation that forskolin treatment enhanced the transcriptional activities of the genes encoding S100b and syncytin B in GCM1-transfected cells but not in control cells is in agreement with the previous finding that forskolin induces protein kinase

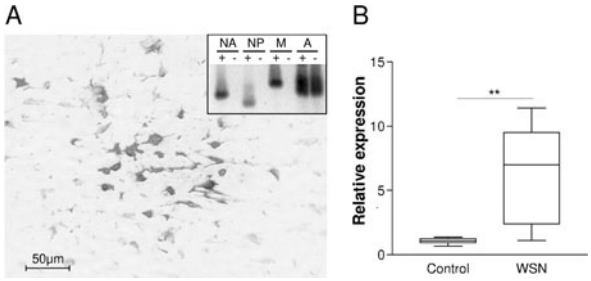

Figure 6 Immunohistochemical detection of influenza A/WSN/33 and levels of transcripts encoding Gcm1 in mouse brain. (**A**) In brains from mice sampled 4 days after i.p. injection of 2400 PFU of influenza A/WSN/33 virus, positive staining of neurons was observed in the motorcortex and amygdala. The insert shows RT-PCR products corresponding to segments 5 (NP), 6 (NA), and 7 (M) of the influenza A/WSN/33 virus and ß-actin. (**A**) (+), virus infected brain tissue; (−), control brain tissue. (**B**) Levels of transcripts encoding Gcm1 in brain tissue from mice sampled 4 days after i.p. injection of PBS or 2400 PFU of influenza A/WSN/33 virus. The levels of transcripts in infected brain tissues are normalized to those observed in control brain tissues. The box-and-whisker plot represents data from seven brains in each group. The box extends from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and lowest values $^{**}P < .01$.

A–mediated stabilization of the GCM1 protein by CREB-binding protein (CBP)-mediated acetylation of GCM1 and thereby increases its activity (Chang *et al*, 2005). Knerr and coworkers (Knerr *et al*, 2005) previously reported that forskolin-treatment of human choriocarcinoma cells or primary trophoblast cells induced transcription of the gene encoding GCM1. This did not appear to be the case in the NIH-3T3 cells investigated here because Gcm1-encoding transcripts remained at the levels observed in control cells following forskolin treatment. The levels of transcripts from the gene encoding syncytin A were not affected by either GCM1 overexpression or forskolin treatment, whereas the combination of the two significantly elevated the levels of such transcripts. The role played by Gcm1 and other cAMP-dependent factors in regulating syncytin A expression remains to be established.

Our finding that the influenza A virus infection can induce transcription of Gcm1 in both primary neurons and glia as well as in whole brain preparations *in vivo* suggests a mechanism by which viral agents may interfere with the development of the brain and induce persistent changes in gene expression long after the infection has been cleared (Asp *et al*, 2005). According to our current *in vivo* data, Gcm1 is transcriptionally active at the time point when the mice were infected; whether ectopic expression of Gcm1 can be induced by a virus infection at later time points is, however, not known. The acute and long-term effects of such insult are the subject of our current studies.

To understand the role of the GCM family of transcription factors in the development of the mammalian brain, additional target genes need to be identified. Other outstanding questions are of course to identify the physiological roles of syncytin A and syncytin B in the murine brain. Furthermore, the pathways activated by the virus infection that are responsible for inducing transcription of Gcm1 remain to be determined. Identification of such pathways will most likely also shed light on the mechanisms regulating expression of GCM1 during physiological and pathological conditions, which so far remain unknown. Although expression of GCM1 in the human brain has not been studied to date, it is interesting to note that increased levels of syncytin mRNA and protein have been detected in brains from patients with multiple sclerosis (Antony *et al*, 2004; Perron *et al*, 2005). Our current findings therefore suggest that the mouse can be used to model the role of GCM1/syncytin not only in the placenta but also in the brain.

Material and methods

Animals and primary cell cultures

C57BL/6 mice were bred at the Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden, and handled according to institutional guidelines. For preparation of primary cell cultures, embryos from pregnant mice were used. The experiments were performed in accordance with permissions from the regional ethical committee for animal experiments (N34/05 and N264/05).

Hippocampal cultures were established as described previously (Owe-Larsson *et al*, 1997). Briefly, hippocampi from E16 embryos (day of vaginal plug = E0) were dissected, collected, and dissociated in 0.1% trypsin (Gibco, Paisley, Scotland, UK). The cells were plated on poly-L-lysine- (Sigma, St. Louis, MO) coated 35-mm tissue culture plates (Corning, New York, USA) at 3–4 $\times$ 10^5 cells/dish and cultured in Neurobasal medium supplemented with B27, 2 mM L-glutamine, and gentamicin (all from Gibco). The medium was not changed prior to experiments, which were performed after 7 days of culture. Glial cell cultures were established by dissecting cortex from E16 embryos and processing the tissue as described above. Cells were cultured in 1:1 Dulbecco's modified Eagle's medium (DMEM):F-12 medium with the following additives: G-5 Supplement (1$\times$), 10% fetal bovine serum, penicillin G sodium (40 U/ml)/streptomycin sulfate (40 μg/ml) all from Gibco. The glial cell cultures were used after two to three passages, a time point at which cells stained positive for the glial marker glial fibrillary acidic protein.

Brain samples

Whole brains were sampled at each of the following time points: embryonal day (E)17 and on postnatal days (P)7, P13, and P24. The brains were removed and snap-frozen on dry ice. From two animals, the hippocampus, cortex, and cerebellum were dissected from freshly prepared brains at each of the following time points: P3, P10, P15, and P27.

Influenza A/WSN/33 infections of cultured cells

Influenza virus A/WSN/33 was obtained from Dr. S Nakajima (The Institute of Public Health, Tokyo, Japan), and propagated on MDCK cell monolayers in MEM supplemented with 0.2 mM L-glutamine, 0.1 M Hepes buffer (Gibco), 0.2% bovine serum albumin (BSA) (Sigma), and penicillin G sodium (50 U/ml)/streptomycin sulfate (50 μg/ml) (Gibco). The virus titer was 2.4 $\times$ 10^7 PFU/ml, as determined by plaque assays on MDCK cells in the absence of trypsin (Tobita *et al*, 1975). For infection with influenza A/WSN/33 virus, cell cultures were washed twice with MEM before addition of virus diluted in MEM (0.5 multiples of infection, MOI). The cultures were carefully agitated every 10 min and after 1 h at 37°C in 5% CO_2, virus supernatants were removed, and complete cell culture medium was added. The infection in glial cells and NIH-3T3 cells (kindly provided by Dr Martin Rottenberg, Karolinska Institutet) were allowed to proceed for 24 h and in hippocampal neurons for 48 h in a humidified 5% CO_2 incubator at 37°C.

Influenza A/WSN/33 infection of mice
C57BL/6 mice were infected intraperitoneally with 2400 PFU of influenza A/WSN/33 virus suspended in 30 μl of PBS on postnatal day 3. Control animals were injected similarly with PBS. In brains from animals sampled at P7, the presence of RNA corresponding to segments 5, 6, and 7 of the influenza A/WSN/33 virus genome were determined by RT-PCR and viral antigens in the brains were analyzed by immunohistochemistry (see below).

Immunostaining
For immunohistochemical labeling of influenza A antigens, series of 12-μm coronal cryostat sections were incubated with a rabbit polyclonal anti-WSN serum (1:1000; a gift from Dr. S Nakajima) and subsequently with a secondary biotinylated swine anti-rabbit immunoglobulin G (IgG) (1:400; DAKO A/S, Denmark). Immunopositivity was visualized after incubation with avidin-biotinylated horseradish peroxidase followed by incubation in sodium acetate buffer containing hydrogen peroxide and 3-amino-9-ethylcarbazole, as previously described (Aronsson *et al*, 2001).

Cloning and transfection of human GCM1
Bases 115 to 1551 in sequence NM_003643, spanning the complete ORF encoding glial cells missing 1 (GCM1), were amplified from a commercially available human placental cDNA (Ambion, Austin TX) using the sense primer 5′-GGCCGA-TCCAGCTATATCAA-3′ and the antisense primer 5′-CTGGGGTGCACATAGTGAAA-3′. One-microliter cDNA template was added to a 50-μl reaction containing 1× Advantage 2 Polymerase Mix (Clontech, Mountain View, CA) and subjected to 20 cycles of amplification a Gen Amp PCR system 9700 (Applied Biosystems, Palo Alto, CA) with the following conditions: initial heat activation at 94°C for 2 min followed by 20 cycles of 94°C for 30 s, 68°C for 2 min, with a final extension of 7 min at 68°C. Amplified fragments were TA-ligated into the pcDNA3.1/V5-HIS-TOPO plasmid (Invitrogen, Carlsbad, CA). Recombinant plasmids were propagated in TOP10 cells (Invitrogen). Plasmids with insert in sense and antisense orientations were purified with the Endo-free plasmid maxi kit according to instructions from the manufacturer (Qiagen, Hilden, Germany) and sequenced at KIseq (Karolinska Institutet, Stockholm, Sweden). NIH-3T3 cells were transfected using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Some cultures were transfected with the pEGFP expression plasmid (Clontech) encoding a variant of green fluorescent protein to control for unspecific effects of protein overexpression. In order to study the influence of cAMP-dependent factors, some cultures were treated with forskolin (100 μM; Sigma-Aldrich) dissolved in DMSO (final concentration 0.1%) for 24 h.

Extraction of RNA and reverse transcription
Total RNA was extracted using the RNeasy kit (Qiagen). The amount and purity of the RNA was assessed by spectrophotometry (Ultrospec Plus, Pharmacia LKB Biotechnology). Total RNA (200 ng to 1 μg) was subsequently treated with 0.5 to 1 unit of amplification grade DNase I (Invitrogen) for 15 min at room temperature and inactivated by the addition of 2.5 mM EDTA followed by incubation at 65°C for 10 min according to the manufacturer's instructions. The DNase-treated RNA was reverse transcribed in 20-μl reactions containing the following reagents from Invitrogen: 150 ng of random hexamers, 1× First Strand Buffer, 10 mM dithiothreitol (DTT), and 500 μM of each dNTP and 100 U Superscript II. cDNA synthesis was allowed to proceed for 1 h at 42°C before inactivation at 72°C for 10 min.

Table 1 Transcripts analyzed by Real-time PCR and RT-PCR, accession numbers, and primer sequences

Target	Accession numbers (position, strand)	Primer sequence, 5′ to 3′
Gcm1	NM_008103	AGTAGACAAGGTCTTCTCACTCAAGCT GGCACGGTGACTTTCTATTCCT
Syncytin A	AF289664 (77413-79263, −)	GAAGAGTGCTGCTTCTACATAAACCA GAGTTCCGAGGCCCTTTCTC
Syncytin B	AC134575 (80271-82124, +)	CCACATCCTACGTGATACCAACTC CCGCTGGGTTTGGGTAGAC
S100b	NM_009115	TGGTTGCCCTCATTGATGTCT TTCTGACTTCTTCAGTTGTGCTT
GAPDH	NM_001001303	TGCACCACCAACTGCTTAGC CAGTCTTCTGAGTGGCAGTGATG Probe: TGGAAGGGCTCATGACCACAGTCCA
Segment 5 of WSN/33 (NP)	AF306656	GAATGGACGGAGAACAAGGA ACGGCAGATCCATACACACA
Segment 6 of WSN/33 (NA)	L25817	ATCTCTTTGTCCCATCCGTG ACCAAGCAACCGATTCAAAC
Segment 7 of WSN/33 (M)	L25818	CAGAGGCCATGGATATTGCT CTCTGGCACTCCTTCCGTAG

RT-PCR

One-microliter cDNA template was amplified in a 20-μl reaction containing 0.5× Titanium Taq DNA Polymerase, 1× PCR Buffer (Clontech), 1.25 μM gene-specific forward and reverse primers (Table 1) and 200 μM of each dNTP (Invitrogen). A GenAmp PCR system 9700 (Applied Biosystems) was used. PCR products were electrophoresed in 2% agarose gel. Double-stranded DNA was stained in 1× SYBR Gold (Invitrogen) and subsequently visualized and documented on a Gel Doc 2000 system (BioRad, Hercules, USA).

Real-time PCR and data analysis

One-microliter cDNA templates were added to triplicate 25-μl reaction mixtures using Platinum SYBR Green qPCR Supermix UDG (Invitrogen) or TaqMan Universal PCR Master Mix (Applied Biosystems) reagents. An ABI Prism 7000 real-time thermocycler was used for all assays. Primers (Invitrogen) and probe (Applied Biosystems) are provided in Table 1. Threshold cycle (Ct) values from the exponential phase of the PCR amplification plot for each target transcript were normalized to that encoding glyceraldehyd-3-phosphate dehydrogenase (GAPDH). From these values, fold-differences in the levels of transcripts between the two groups were calculated according to the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).The non-parametric Mann-Whitney test was used to compare the relative levels of transcripts between groups.

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